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Julie A. Preston[1†], Martin A. Bewley[1†] , Helen M. Marriott[1†], A. McGarry Houghton[2], Mohammed Mohasin[1], Jamil Jubrail[1], Lucy Morris[1], Yvonne L. Stephenson[1], Simon Cross[1], David R. Greaves[3], Ruth W. Craig[4], Nico van Rooijen[5], Colin D. Bingle[1], Robert C. Read [6], Timothy J. Mitchell[7], Moira K.B. Whyte[8], Steven D. Shapiro[9] and David H. Dockrell[10]*

[1] The Florey Institute for Host-Pathogen Interactions and Department of Infection, Immunity and Cardiovascular Disease, University of Sheffield Medical School, S10 2RX, UK,[2]Division of Pulmonary, Allergy and Critical Care Medicine, University of Pittsburgh Medical Center, USA, [3]Sheffield Teaching Hospitals, S10 2RX, UK
[3] Sir William Dunn School of Pathology, University of Oxford, OX1 3RF, UK
[4]Department of Pharmacology and Toxicology, Geissel School of Medicine at Dartmouth, 03755, NH, USA,[5] Department of Molecular Cell Biology and Immunology, VU University Medical Center, Amsterdam, 1081 HZ, The Netherlands, [6]University of Southampton Medical School and NIHR Southampton Biomedical Research Centre, Southampton, SO166YD, UK, [7] Institute of Microbiology and Infection, School of Immunity and Infection, University of Birmingham, B15 2TT, UK Trust, M23
[8]Department of Respiratory Medicine and MRC Centre for Inflammation Research, University of Edinburgh. [9]Division of Pulmonary, Allergy and Critical Care Medicine, University of Pittsburgh Medical Center, USA, [10] Department of Infection Medicine and MRC Centre for Inflammation Research, University of Edinburgh Corresponding **Author:** David H. Dockrell, The MRC/University of Edinburgh Centre for Inflammation Research, 47 Little France Crescent, Edinburgh, Edinburgh EH16 4TJ UK Phone: +44 (0) 131 242 658 Fax: +44 (0) 131 242 6578 email: david.dockrell@ed.ac.uk.
† These authors contributed equally.

Author contributions: JAP, MAB and HMM contributed equally to this work and generated figures. JAP made and validated the transgenic mouse and performed *in vivo* infections. MAB performed killing assays, flow cytometry, collected data and produced figures. HMM performed *in vivo* experiments involving bone marrow transplantation, neutrophil depletion and designed and conducted experiments involving therapeutic targeting. MH helped design and conduct experiments to generate the transgene construct. DRG designed the CD68 construct. RWC designed the Mcl-1 construct. CDB helped in design of the targeting vector and experiments to evaluate its expression. JJ performed experiments with *S. aureus*. LM performed analysis of BMDM phenotype. YLS and SC performed histopathology. NR provided expertise in liposome experiments. RCR and TJM helped design infection models. JAP, MAB, HMM, MKBW, SS and DHD designed and conceived the experiments. JAP, MAB, HMM, MKW, DRG, RWC, and DHD wrote the manuscript with input from all other authors.

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At a glance summary:

Scientific Knowledge on the Subject: The exact mechanisms used by alveolar macrophages (AM) to kill extracellular bacteria remain unclear.
What This Study Adds to the Field: We have generated a novel transgenic mouse with AM which over-expresses the anti-apoptotic factor Mcl-1, a molecule that is over-expressed in several patient groups at increased risk of pneumonia, which demonstrates this transgenic has a reduced capacity to clear bacteria from the lung. Apoptosis-associated killing is activated when initial phagolysosomal mechanisms are exhausted and requires a combination of reactive species, including nitric oxide and mitochondrial-derived reactive oxygen species. Re-engaging apoptosis when deficient pharmacologically helps prevent pneumonia in these murine models.

This article has an online data supplement, which is accessible from this issue's table of content online at **www.atsjournals.org**

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Abstract

Rationale: Antimicrobial resistance challenges therapy of pneumonia. Enhancing macrophage microbicidal responses would combat this problem but is limited by our understanding of how alveolar macrophages (AM) kill bacteria.

Objectives: To define the role and mechanism of AM apoptosis-associated bacterial killing in the lung.

Methods: We generated a unique CD68.hMcl-1 transgenic mouse with macrophage-specific over-expression of the human anti-apoptotic Mcl-1 protein, a factor upregulated in AM from patients at increased risk of community-acquired pneumonia, to address the requirement for apoptosis-associated killing.

Measurements and Main Results: Wild-type and transgenic macrophages demonstrated comparable ingestion and initial phagolysosomal killing of bacteria. Continued ingestion (for $\geq$ 12 h) overwhelmed initial killing and a second late-phase microbicidal response killed viable bacteria in wild-type macrophages, but this response was blunted in CD68.hMcl-1 transgenic macrophages. The late-phase of bacterial killing required both caspase-induced generation of mitochondrial reactive oxygen species (mROS) and nitric oxide (NO), whose peak generation coincided with the late-phase of killing. The CD68.hMcl-1 transgene prevented mROS but not NO generation. Apoptosis-associated killing enhanced pulmonary clearance of *Streptococcus pneumoniae* and *Haemophilus influenzae* in wild-type but not CD68.hMcl-1 transgenic mice. Bacterial clearance was enhanced *in vivo* in CD68.hMcl-1 transgenic mice by reconstitution of apoptosis with BH3 mimetics or clodronate-encapsulated liposomes. Apoptosis-associated killing was not activated during *Staphylococcus aureus* lung infection.

Conclusions: Mcl-1 upregulation prevents macrophage apoptosis-associated killing and establishes that apoptosis-associated killing is required to allow AM to clear ingested

bacteria. Engagement of macrophage apoptosis should be investigated as a novel host-based antimicrobial strategy.

Introduction

Community-acquired pneumonia (CAP), commonly caused by *Streptococcus pneumoniae* (the pneumococcus) and other bacteria, is a leading causes of global mortality (1). The plasticity of bacterial genomes challenges vaccination and facilitates antimicrobial resistance (2). Pathogenic bacteria frequently colonize the upper airway, but CAP is relatively uncommon, indicating efficient host responses protect most individuals.

Tissue macrophages, such as alveolar macrophages (AM), are key effectors of antibacterial host defense (3), but the mechanisms used to kill extracellular bacteria after their internalization are incompletely defined. AM kill ingested bacteria in phagolysosomes, but this mechanism is less efficient than in other phagocytes. Tissue macrophages usually do not express myeloperoxidase (4) or the microbicidal serine proteases seen in neutrophils (5) and are less reliant on nicotinamide adenine dinucleotide phosphate (NADPH) oxidase-dependent reactive oxygen species (ROS) generation (6). Nitric oxide (NO) generation in human macrophages is also less vigorous than in rodent cells or monocytes (7, 8). Moreover, pneumococci and other bacterial pathogens frequently express genes that inhibit phagolysosomal killing (9). Prolonged intracellular killing of bacteria is associated with macrophage apoptosis in human macrophages and in murine pneumonia models (3, 10). Although inhibition of apoptosis reduces bacterial killing in these murine models it has not been demonstrated if cell-autonomous macrophage apoptosis mediates pathogen clearance (3, 11). Recently we have found that a key regulator of macrophage apoptosis during bacterial killing, the anti-apoptotic protein Mcl-1 (11), is upregulated in AM from patients at increased risk of CAP due to chronic obstructive pulmonary disease (COPD) or HIV-1 infection, where it is associated with reduced AM apoptosis and bacterial killing ex vivo (12, 13). Re-engaging microbicidal responses downstream of apoptosis restored bacterial killing in COPD AM (12) but

whether apoptosis reconstitution in the presence of over-expression of Mcl-1 restores bacterial killing is unknown.

To test if macrophage cell autonomous over-expression of the human Mcl-1 transgene, as observed in these patients at increased risk of CAP, mediates bacterial clearance we generated transgenic mice that specifically express CD68.hMcl-1 in macrophages, since the viability of these cells is closely linked to expression of this anti-apoptotic protein (11, 14). We used this novel transgenic line with controlled infections and interventions to define the role, microbicidal mechanism and potential for therapeutic re-engagement of macrophage apoptosis-associated bacterial killing. Our findings show that macrophage apoptosis represents a second late-phase of bacterial killing, which is activated after initial lysosome-mediated mechanisms are exhausted upon sustained bacterial uptake. Apoptosis-associated bacterial killing requires mitochondrial (mROS), which act in combination with NO. This microbicidal mechanism was inhibited in the presence of CD68.hMcl-1, but was restored by BH3 mimetics or bisphosphonates.

Materials and Methods

Generation of CD68.hMcl-1 transgenic mice: A 1.5kb fragment containing the cDNA sequence for human Mcl-1 (15) was cloned into a plasmid containing 2.9 kb of the CD68 promoter with the first intron enhancer IVS (16) (Figure 1A). Correct orientation and PCR mismatches were confirmed by sequencing. The transgene was isolated by restriction enzyme digestion and gel purification. The transgene was microinjected into C57Bl/6J oocytes (Washington University School of Medicine, St Louis, USA). Founders and their progeny were genotyped by PCR amplification of tail or ear biopsy DNA using the following primers: 5'-ACCATCTCCTCTCTGCCAAA-3' and 5'-GGGCTTCCATCTCCTCAA-3'. Two CD68.hMcl-1 mice transgenic lines were established. Both lines showed germline transmission and equivalent functional results. Mcl-1 transgenic mice and non-transgenic littermates, from the same cages, were inoculated with bacteria and sample collected as described previously (3) and in the on-line supplement.

Bacteria: Details on the bacteria and culture conditions are provided on-line.

Isolation and culture of macrophages and other leukocytes: Bone marrow-derived macrophages (BMDM), resident AM , peritoneal macrophages (PM), peripheral blood neutrophils, B-cells and T-cells were obtained from C57Bl/6J mice (17, 18). Human monocyte-derived macrophages (MDM) from whole blood donated by healthy volunteers (10). Further details are available on-line.

Flow Cytometry and confocal microscopy: Further details on flow cytometry and confocal microscopy experiments are available in the on-line supplement.

Intracellular killing assay: Assessment of intracellular bacterial viability was carried out as previously described (19), and outlined in the on-line supplement.

Reconstitution of Apoptosis: Apoptosis was reconstituted in vitro with clodronate-encapsulated liposomes or the indicated BH3 mimetics and in vivo AM apoptosis was reconstituted in transgenic mice using clodronate containing liposomes or the indicated BH3 mimetics (further information in the on-line supplement), with instillation of bacteria at the same time to ensure induction of early-stage apoptosis but not macrophage depletion (20).

Ethics: Animal experiments were conducted in accordance with the Home Office Animals (Scientific Procedures) Act of 1986, authorized under a UK Home Office License 40/3251 with approval of the Sheffield Ethical Review Committee. MDM were isolated from healthy volunteers with written informed consent and approval from the South Sheffield Regional Ethics Committee.

Statistics: Results are recorded as mean and SEM. Sample sizes were informed by standard errors obtained from similar assays in prior publications (10, 11). D'Agostino-Pearson normality tests guided test selection. Comparisons between two conditions were performed using a paired or unpaired t-test for parametric data, or a Mann-Whitney U test or Wilcoxon signed rank test for non-parametric data using Prism 6.0 software (GraphPad Inc.). Comparisons between three or more conditions were performed using a normal or

repeated measures 1-way ANOVA with Bonferroni post-test for parametric data, or a Friedman test with Dunn's multiple comparison post-test for non-parametric data. When two or more conditions were assessed in two experimental groups data was analysed by 2-way ANOVA with Bonferroni post-test. Significance was defined as $P < 0.05$.

Results

CD68.hMcl-1 transgenic mice demonstrate reduced macrophage apoptosis

A CD68 promoter construct (21) ensured macrophage-specific human Mcl-1 (hMcl-1) expression, to generate a transgenic mouse with selective apoptosis resistance in macrophages (Figure 1A). Equivalent functional results were generated from both founder lines. Macrophage-specific hMcl-1 expression was documented in AM and other macrophage lineages in CD68.hMcl-1 transgenic mice (Figure 1B-D), but not neutrophils or lymphocytes (Figure 1E). CD68.hMcl-1 transgenic mice lacked a gross developmental phenotype or loss of fertility and showed normal survival. CD68.hMcl-1 mice had normal numbers of leukocyte subsets in blood and of macrophage in tissues, while splenic lymphoid tissue and lung parenchyma showed no histological abnormalities (Figure E1A-F).

Importantly, the transgene reduced susceptibility to apoptosis in bone marrow-derived macrophages, (CD68.hMcl-1^+ BMDM), AM and peritoneal macrophages (Figure 1F-H and E1G). In contrast, CD68.hMcl-1^+ BMDM demonstrated no decrease in binding or ingestion of latex beads or in the numbers of viable intracellular bacteria present after 4 h exposure to *S. pneumoniae* (a marker of early ingestion and killing (10)) (Figure E1H-I). The generation of ROS and NO was also unaltered by the transgene at 4 h (Figure E1J-K). Mcl-1 thus did not alter initial innate immune responses.

Mcl-1 expression regulates apoptosis-associated killing when phagolysosomal bacterial killing is exhausted

Exposure of macrophages to pneumococci for 16-20 h results in apoptosis without a loss of membrane integrity (22). Importantly, this was inhibited in the presence of the

CD68.hMcl-1 transgene, which acted at the level of the mitochondrial execution of the program of apoptosis (Figure E2A-D). The transgene also increased survival of ingested bacteria (Figure 2A), although it had no effect on lysosomal acidification, lysosomal membrane permeabilization, or activation of the lysosomal protease cathepsin D, which occurs upstream of the mitochondrial apoptotic program following pneumococcal challenge (11, 14) (Figure E2E-G). To prove that reduction of macrophage apoptosis was the mechanism by which Mcl-1 inhibited bacterial killing, apoptosis was reconstituted with the BH3 mimetic ABT-737. Although ABT-737 cannot reverse the anti-apoptotic effect of Mcl-1 directly, it interacts with Bcl-2/Bcl-X_L, displacing pro-apoptotic Bcl-2 proteins to stimulate apoptosis (23). ABT-737 increased the number of cells with loss of inner mitochondrial transmembrane potential ($\Delta\psi_m$) and nuclear fragmentation in CD68.hMcl-1^+ BMDM exposed to pneumococci (Figure E3A-B), and restored apoptosis-associated intracellular bacterial killing (Figure 2B), while additional BH3 mimetics also increased bacterial killing at 20 h, but not at 4 h (Figure E3C-D).

To dissect the role of apoptosis-associated killing in host defense, BMDM were ‘pulsed’ with bacteria and the kinetics of killing of internalized bacteria were measured following antimicrobial ‘chase’ to remove extracellular bacteria. The ‘chase’ does not itself alter internalized bacteria since the cell membrane remains intact at this early-stage of apoptosis (22) and ensures that changes in viable bacteria are the result of intracellular killing but not continued phagocytosis. We identified two phases of intracellular killing. An initial-phase, occurring immediately after bacterial ingestion (Figure 2C), was consistent with phagocytosis-associated phagolysosomal killing (24) and was similar in transgenic and non-transgenic BMDM. A late-phase of intracellular killing occurred at 16-20 h in non-transgenic BMDM but was blunted in CD68.hMcl-1^+ BMDM. This late-phase coincided with the onset of apoptosis (Figure 2D) but occurred prior to any

reduction in macrophage cell numbers (Figure 2E). By varying the duration of the 'pulse' we confirmed that the initial phase of bacterial killing was sustained for up to 12 h and that, after it ceased, the late-phase of killing cleared viable internalized bacteria (Figure 2F). Overall, the CD68.hMcl-1 transgene inhibited the late-phase of bacterial killing without any impact on early ingestion or killing.

We tested whether bacterial ingestion was compromised after $\geq$12 h of exposure to bacteria, as this would remove the stimulus for phagolysosomal killing. We investigated this using a second 'pulse' with a distinct strain of bacteria (Figure 2G). Bacterial internalization occurred at $\geq$12 h in the presence or absence of the Mcl-1 transgene, but was accompanied by continued intracellular killing only in CD68.hMcl-1^{-} BMDM. Sustained bacterial ingestion activated a late-phase of killing, requiring apoptosis induction, killing viable internalized bacteria.

Mitochondrial ROS is required for apoptosis-associated bacterial killing

An inhibitor of nitric oxide synthase (NOS) 2 reduced both the late-phase of bacterial killing and apoptosis in non-transgenic BMDM (Figure 3A-B). This suggested that NO generation contributed to bacterial killing, but occurred upstream of apoptosis, consistent with its known role in sensitizing mitochondria to apoptosis induction (19). An antioxidant also inhibited late-phase bacterial killing, although it did not affect apoptosis-induction. This suggested that ROS are also required for this phase, but are generated downstream or as a result of apoptosis. Since NADPH oxidase does not contribute to macrophage apoptosis-associated killing during pneumococcal infection (25), we addressed another source of antimicrobial ROS, mitochondrial ROS (mROS) (26).

Increased mROS were apparent after 16-20 h of bacterial exposure, but were reduced by the Mcl-1 transgene (Figure 3C). Peak generation of mROS co-incided with peak NO production in human MDM (Figure E4A) and mROS/NO co-localized with bacteria (Figure E4-6). Since mROS generation was a late response contemporaneous with apoptosis onset we tested whether caspase 3 activation, which inhibits mitochondrial electron transport complex I and II, contributed to mROS generation (27). Caspase activation increased mROS production and, consistent with the role of Mcl-1 in limiting caspase activation, the caspase $3/7^{+}$ population was expanded in non-transgenic versus transgenic BMDM and produced significantly more mROS after 20 h of exposure to bacteria (Figure 3D). Comparable findings were observed in human MDM (Figure 3E). Crucially, an inhibitor of mROS, mitoTEMPO, blocked the late-phase of pneumococcal killing in CD68.hMcl-1^{-} (but not CD68.hMcl-1^{+}) BMDM (Figure 3F) and also in human MDM (Figure 3G). Inhibition of mROS did not modify BMDM apoptosis-induction or Mcl-1 expression (Figure 3H and 3I), consistent with mROS acting downstream of apoptosis in bacterial killing. Since mROS also activates pro-inflammatory cytokine expression (28) we confirmed differential cytokine expression was not contributing to differences in late microbicidal responses (Figure E7 A-C). Since pneumococci intrinsically resist oxidative stress (29), our results suggest apoptosis-associated killing requires caspase-dependent mROS generation, combined with NO, to mediate bacterial killing

Apoptosis-associated killing is required for bacterial clearance in vivo

We next addressed the role of apoptosis-associated bacterial killing by AM *in vivo.* Initially we used a low-dose of pneumococci which AM clear efficiently, an intermediate dose, which represents the ‘tipping-point’ at which AM start to fail to control infection and where any increase in dose or perturbation of macrophage function results in

development of pneumonia, and a high doses where AM are overwhelmed and mice develop systemic infection (3, 30). CD68.hMcl-1^{+} transgenic mice failed to clear the low dose of pneumococci (10^{4} colony forming units; CFU) by 24 h, while non-transgenic mice cleared all bacteria (Figure 4A) (3). Only transgenic mice developed bacteremia at the low dose (Figure 4B). At intermediate doses, CD68.hMcl-1^{+} transgenic mice also exhibited increased bacterial CFU in lungs and blood compared to non-transgenic mice. Crucially the transgenic mice had significant neutrophil recruitment in BAL at this intermediate dose, a feature of pneumonia, while the non-transgenic animals had no neutrophil recruitment (Figure 4C). AM numbers were not altered by low dose infection or transgene expression but were only reduced in the high dose infection in transgenic mice in association with high-levels of inflammatory cell recruitment (Figure 4D). The bacterial clearance that occurred in non-transgenic animals was completely overwhelmed as expected at an inoculum of 10^{7} CFU macrophages (3). Along with reduced bacterial clearance and an increased requirement for neutrophil recruitment, CD68.hMcl-1^{+} transgenic mice exhibited reduced AM apoptosis in bronchoalveolar lavage (BAL) following bacterial challenge (Figure 4E). To exclude any role for low numbers of lung neutrophils in the differential clearance of bacteria we repeated low dose bacterial challenge after neutrophil depletion and again confirmed reduced bacterial clearance in the transgenic mice (Figure E8). Overall this proved the transgene reduced bacterial replication and also the threshold at which neutrophils were recruited to control bacteria in the lung, but only during the specific stages where AM are the major effector of bacterial clearance in the lung.

CD68.hMcl-1^{+} transgenic mice also exhibited impaired clearance of low doses of *H. influenzae*, another respiratory pathogen (31). At high doses, infection progressed to pneumonia with pulmonary neutrophil recruitment in all mice, since AM clearance

capacity was overwhelmed (Figure 4F-G). Reduced bacterial clearance at low doses was also associated with reduced macrophage apoptosis in the BAL (Figure 4H). Apoptosis-associated killing likewise contributed to bacterial clearance at extra-pulmonary sites: CD68.hMcl-1^{+} transgenic mice given a low peritoneal dose of *S. pneumoniae* showed impaired peritoneal clearance of bacteria, increased numbers of bacteria in blood, enhanced neutrophil numbers and reduced macrophage apoptosis (Figure 4I-L).

Re-engagement of macrophage apoptosis enhances pulmonary bacterial clearance in vivo

To confirm that the differential levels of apoptosis explained the transgene effect *in vivo*, we reconstituted AM apoptosis in the CD68.hMcl-1^{+} transgenic mice using liposomes containing clodronate (20). Liposomes ensure macrophage targeting through phagocytic uptake, while clodronate induces a mitochondrial pathway of apoptosis with loss of $\Delta\psi_m$ providing an alternative route of engagement of the mitochondrial apoptosis pathway in the absence of Mcl-1 downregulation (32, 33). Liposome dosing was adjusted to induce apoptosis in CD68.hMcl-1^{+} BMDM exposed to bacteria (Figure 5A), without altering bacterial internalization (Figure 5B). *In vivo* we administered liposomes at the same time as bacteria, to ensure that the early stages of liposome-induced AM apoptosis occurred together with the initiation of the anti-bacterial apoptotic program and before AM depletion reduced AM numbers (Figure 5C and 5D). Reconstitution of AM apoptosis in CD68.hMcl-1^{+} AM increased bacterial clearance from the lung, reduced levels of bacteria in the blood, and reduced neutrophil numbers in BAL (Figure 5E-G). Similar results were obtained with BH3 mimetics (ABT-263, an oral derivative of ABT-737 and sabutoclax, a pan Bcl-2 family inhibitor (34)). These increased bacterial clearance from lung and blood (Figure 5H-I) and AM apoptosis but not AM numbers, in infections inducing minimal neutrophil recruitment (Figure E9). They also reduced viable bacteria in MDM at late, but not early time-points (Figure E9D-E).

Adoptive transfer of bone marrow between non-transgenic and transgenic mice confirmed our results reflected macrophage expression of the transgene. Bone marrow transplantation reduces the ability of mice to clear the low dose of pneumococci (14, 35) and bacteria were not completely cleared in either group of mice (Figure E10A-B). However, the recipients of transgenic bone marrow exhibited reduced AM apoptosis and greater numbers of macrophage-associated bacteria (consistent with reduced intracellular clearance), while recruiting significantly more neutrophils (Figure E10C-E). This suggested that there was less effective macrophage killing in transgenic mice.

Staphylococcus aureus infection does not activate apoptosis-associated killing

Staphylococcus aureus upregulates Mcl-1 in macrophages (36). We wondered whether this would phenocopy the effects seen with the CD68.hMcl-1 transgene. Induction of macrophage apoptosis requires downregulation of Mcl-1 to allow mitochondrial outer membrane permeabilization (MOMP) and the execution phase of apoptosis (11, 14). *S. aureus* failed to induce the anticipated Mcl-1 downregulation after sustained bacterial ingestion (Figure E11A). Moreover, it was not associated with apoptosis or late-phase bacterial killing (Figure 6A-B), despite exhaustion of the initial phase of killing in the setting of sustained ingestion of bacteria (Figure 6C-D). Exposure *in vivo* to a range of bacterial doses (from doses AM can control to 100-fold higher) did not reveal any differences in bacterial clearance, neutrophil recruitment, or AM apoptosis irrespective of transgene expression (Figure 6E-G). In contrast to pneumococcal infection, ABT-737 failed to enhance bacterial killing or to reconstitute apoptosis at the dose that induced apoptosis in transgenic BMDM after pneumococcal challenge (Figure E11B-C). ABT-737 was used to reconstitute apoptosis since it does not alter uptake, in contrast to

liposomes (3), in which altered phagocytosis can confound interpretation during high-uptake phagocytosis, as seen with *S. aureus*. These findings highlight the importance of apoptosis-associated killing as a mechanism subverted by some pathogens.

Discussion:

Development of a macrophage specific CD68.hMcl-1 transgenic mouse provided a unique means of examining the role and mechanism of macrophage apoptosis-associated bacterial killing in the lung. Use of this model identified a novel paradigm whereby macrophage apoptosis kills internalized bacteria that remain viable after initial phagolysosomal killing is exhausted. During apoptosis induction caspase-dependent mROS production combines with NO to achieve a second late-phase of bacterial killing. Inhibition of macrophage apoptosis by Mcl-1 increases susceptibility to bacterial infection but can be modulated pharmacologically to enhance pulmonary bacterial clearance.

Macrophages' avid phagocytic capacity ensures intracellular loading with ingested bacteria (37, 38). Phagocytosis activates an initial-phase of bacterial killing, consistent with observations describing temporal association of the NOX2 complex with neutrophil phagocytosis (24), but sustained phagocytosis overwhelms initial microbicidal responses. A late-phase microbicidal response, during the initial stages of apoptosis, clears remaining viable internalized bacteria. Macrophage apoptosis occurs during *M. tuberculosis* infection (39), but also with other pulmonary micro-organisms, such as pneumococci, unable to persist intracellularly, suggesting it limits intracellular persistence (11, 14).

Our approach, using macrophage specific transgene expression (16), allowed selective modulation of the early stages of apoptosis via Mcl-1, with relative resistance to apoptosis, which regulates macrophage survival following pneumococcal infection (11). Mcl-1 is unique amongst anti-apoptotic Bcl-2 proteins because it is an early response gene with rapid induction and turnover (40). Mcl-1 transgene expression in myeloid cells prolongs macrophage survival but ensures sensitivity to physiological constraints on viability, and that cell numbers remain within the normal range (15).

Emerging data in patients at risk of CAP show Mcl-1 upregulation in AM is associated with reduced bacterial killing (12, 13). During HIV-1 infection gp120 inhibits Mcl-1 ubiquitination and proteasomal degradation while in COPD transcriptional upregulation is associated with anti-oxidant responses during oxidative stress (12, 13) . In our murine model over-expression of Mcl-1 using the human transgene converted low dose lung infections, which macrophages normally control (3), into established infections inducing neutrophilic inflammation, phenocopying the susceptibility of patient AM *ex vivo*. In comparison with *S. pneumoniae* and *H. influenzae*, *S. aureus* is less readily killed by differentiated macrophages (41) and internalized bacteria remain viable for several days (42). *S. aureus* containing-phagosomes fail to mature appropriately, decreasing cathepsin D activation required for Mcl-1 proteasomal degradation (14, 43).. We show *S. aureus* prevents apoptosis-associated killing, however unlike pneumococcal infection we could not reconstitute apoptosis-associated killing following *S. aureus* infection. A potential explanation for this finding could be that altered endosomal trafficking of *S. aureus* needs to be corrected (43), to allow induction of apoptosis and to allow co-localization with mitochondria to mediate microbicidal killing (26). Thus HIV, COPD and *S. aureus* infection all inhibit bacterial killing by upregulating Mcl-1, similar to the over-expression of the human Mcl-1 transgene in our murine model.

The relevance of animal models to human disease merits careful scrutiny. Murine pneumonia models confirm roles for the key innate cell populations contributing to pathogenesis in CAP and reprise the susceptibility of key single gene defects or polymorphisms identified in humans, despite some differences in specific innate responses (e.g. extent of reliance on NOS2 (inducible NO synthase) in macrophages, α-defensin expression in neutrophils or activation patterns following specific Toll-like

receptor ligands) (44). The C57Bl/6 strain has intermediate susceptibility to pneumococci (45) and inocula can be adapted to favour AM-dependent clearance or sequential requirement of T-cells and recruited neutrophils in this model (30). They also show evidence of AM apoptosis (3) and increased susceptibility to pneumococcal disease following Mcl-1 over-expression (11). A murine model allowed us to test the impact of genetic modulation of Mcl-1 *in vivo* in the context of early stage sub-clinical infection, something not possible in patients who present at the later stage of established disease. Impaired clearance of pneumococci in association with Mcl-1 upregulation in patient groups at increased risk of CAP suggests these finding are relevant to human disease. Moreover, we demonstrated key mechanistic requirements for caspase-dependent mROS, combined with NO, in apoptosis-associated killing in human MDM, as well as increased bacterial clearance with BH3 mimetics.

Apoptosis-associated bacterial killing requires mROS, a recently identified microbicidal (26). During apoptosis execution caspase 3, inhibits mitochondrial electron transport complexes I and II (27), resulting in in generation of superoxide (46). SOD2 upregulation during pneumococcal infection prevents necroptosis (47, 48) ensuring mitochondrial permeabilization is limited in extent, a specific feature of apoptosis (11, 14, 48, 49). However, antioxidant protection does not extend to the immediate environment of the phagolysosome, permitting microbial killing (26). Pneumococcal anti-oxidant systems protect against NADPH-dependent ROS generation in neutrophils (9). Our results, however, suggest that peak mROS and NO co-exist, consistent with the role of NO in the late microbicidal macrophage response (19, 25). Potential sources of NO include NOS2 but also NOS3 (endothelial NOS), which contributes to AM microbicidal responses to pneumococci (50) and mitochondria which generate NO through NOS-independent and NOS-dependent mechanisms (including the debated existence of an inner membrane-

associated or matrix isoform) (51). Cross-reactivity of inhibitors between isoforms and residual controversies concerning NOS2 in humans means the source of NO requires further clarification. We propose a model where mROS and NO generation is temporally and spatially linked, and co-localizes with bacteria containing phagolysosomes as previously shown (19), allowing generation of reactive nitrogen species (RNS). We did not identify NO regulation by mROS, since mROS occurred downstream of Mcl-1-mediated apoptosis regulation, while NO production was upstream and unaltered by the Mcl-1 transgene. NO and RNS can, however, enhance mROS generation (51). We found no evidence that mROS role in killing was mediated by differential cytokine expression. While mROS can induce pro-inflammatory cytokine expression (28), during the early stages of bacterial-associated apoptosis induction protein translation is reduced, limiting this possibility (36).

Since we demonstrate a critical role for apoptosis-associated killing in mediating bacterial clearance by macrophages, it follows that upregulation of Mcl-1 influences susceptibility to bacterial pneumonia. The use of a murine model in which we could alter Mcl-1 expression through transgene expression and deliver controlled infections and pharmacological interventions allowed us to confirm the role and mechanisms of this process to an extent not possible with our prior studies in patients (12, 13). Our data suggests that susceptibility to bacterial infection can be reversed through therapeutic targeting of the mitochondrial-microbicidal axis or modulation of Mcl-1. Several classes of therapeutics, including bisphosphonates and BH3 mimetics, target these pathways (20, 23). As proof of concept of this repurposing approach ABT737 has recently demonstrated utility in preventing intracellular replication of *Legionella pneumophila* in AM through induction of apoptosis (52). We also demonstrated Bcl-2 specific and pan-Bcl-2 inhibitors enhanced pneumococcal clearance, with more significant results demonstrated for

sabutoclax an agent that inhibits Mcl-1 (53). However, for some pathogens like *S. aureus* the strategy may need to be adapted to reverse altered endosomal trafficking (43). In view of the on-going therapeutic challenge of antimicrobial resistance, re-engaging this fundamental microbicidal mechanism in AM merits further evaluation.

Disclosure of Conflicts of Interest

All authors have declared that no competing financial interests exist.

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Figure Legends

Figure 1: Macrophages from CD68.hMcl-1 transgenic mice express hMcl-1 and have selective resistance to apoptosis.

(A) Schematic representation of the transgene construct. Human Mcl-1 (hMcl-1) expression is driven in macrophages by 2.9 kb of the CD68 promoter and Intron 1 (IVS-1). **(B-D)** Western blot analysis of human (h) and murine (m) Mcl-1 protein expression in (B) bone marrow-derived macrophages (BMDM), (C) alveolar, and (D) peritoneal macrophages, from CD68.hMcl-1 non-transgenic (non-Tg) or transgenic (Tg) mice. **(E)** Peripheral blood neutrophils and splenic $CD19^+$ B-lymphocytes (CD19+) or $CD3^+$ T-lymphocytes (CD3+) were isolated from non-Tg or Tg mice. Cells were lysed and probed by western blot for human (h) Mc-1 protein expression. Blots representative of three independent experiments. The +ve control is human monocyte-derived macrophage lysate. **(F-G)** BMDM from non-Tg or Tg mice were irradiated with UV radiation and assessed for (F) nuclear fragmentation at the indicated time-points, n=4, ***= $p<0.001$, 2-way ANOVA, or (G) caspase 3/7 activation measuring relative luminescence units (RLU) at 8h, n=4, *= $p<0.05$, 2-way ANOVA. Data are represented as mean ±SEM. **(H)** Alveolar (AM) macrophages from non-Tg or Tg mice were left untreated (negative), or UV treated. 8 h after UV exposure, apoptosis was assessed by nuclear fragmentation, n=4-5, ***= $p<0.001$ 2-way ANOVA. Data are represented as mean ±SEM. See also Figure E1.

Figure 2: Apoptosis-associated killing ensures intracellular bacterial clearance when canonical phagolysosomal killing is exhausted.

(A) Bone marrow-derived macrophages (BMDM) from CD68.hMcl-1 non-transgenic (non-Tg) or transgenic (Tg) mice were mock-infected (MI) or challenged with serotype 2 *S. pneumoniae* (Spn) at a multiplicity of infection (MOI) of 10 and intracellular colony

forming units (CFU) assessed 20 h after infection, n=18, **= p<0.01, unpaired Student's t-test. **(B)** Non-Tg or Tg BMDM were challenged with Spn in the presence (+) or absence (-) of ABT-737. 20 h post-challenge cells were assessed for intracellular CFU, n=9, ***= p<0.001, 2-way ANOVA. **(C)** Non-Tg or Tg BMDM were challenged with Spn at MOI of 10 for 4 h and extracellular bacteria removed ('pulse-chase' design). BMDM were lysed for initial assessment of CFU or incubated in vancomycin until the designated time point when CFU were also determined, n=4, **= p<0.01, 2-way ANOVA. **(D-E)** BMDM were challenged with Spn at MOI of 10. At the designated time post-challenge, levels of apoptosis, (D), and the average number of cells per field (E), were measured, n=4, *= p<0.05, 2-way ANOVA**. (F)** Non-Tg or Tg BMDM were challenged with Spn at MOI of 10 for varying times before extracellular bacteria were killed and intracellular CFU estimated immediately or after a further 2 h incubation to measure intracellular killing capacity, n=4, **= p<0.01, 2-way ANOVA. **(G)** Non-Tg or Tg BMDM were challenged with Spn at MOI of 10 for varying intervals before extracellular bacteria were killed and cultures split, and one group were 'pulsed' with streptomycin resistant Spn (FP58) for 2 h, after which the intracellular CFU of FP58 were estimated as a marker of recent ingestion, n=5. Data are represented as mean ±SEM. See also Figures E2-3.

Figure 3. Mcl-1 regulates caspase-induced late-phase mitochondrial ROS production.

(A-B) Bone marrow-derived macrophages (BMDM) from CD68.hMcl-1 non-transgenic (non-Tg) or transgenic (Tg) mice were challenged with serotype 2 *Streptococcus pneumoniae* (Spn) at a multiplicity of infection (MOI) of 10 in the presence (+) or absence of the antioxidant Trolox, or an iNOS inhibitor (1400W). (A) Intracellular bacterial colony forming units (CFU) were estimated 16 h post-challenge, n=5, or (B) nuclear

fragmentation was recorded 20 h post-challenge, n=6, **= p<0.01, 1-way or 2-way ANOVA for analyses within or between groups respectively. **(C)** Non-Tg or Tg BMDM were mock-infected (MI) or challenged with Spn at MOI of 10. At the indicated times post-challenge cells were stained with MitoSOX and analysed by flow cytometry. n=3 *= p<0.05, Students t-test. **(D)** Non-Tg or Tg BMDM were MI or challenged with Spn at MOI of 10. At the designated time points, cells were stained for mROS and caspase 3/7 activity by flow cytometry. Cells were selected by forward/side scatter (grey contour plots), before being designated as caspase negative or positive (green contour plots). MitoSox red staining was assessed for each caspase subpopulation and cell populations as a whole (histograms). Representative plots are shown, with collated data in the graph below, n=3 *= p<0.05, 2-way ANOVA. **(E)** Experiments in D were repeated in human MDM, n=4 *= p<0.05, 1-way ANOVA. **(F-G)** Non-Tg or Tg BMDM (F) or human MDM (G) were challenged with Spn at MOI of 10, in the presence or absence (vehicle) of mitoTEMPO (mT), 1400W, or a combination of both (Combo). 16 h post-challenge intracellular CFU were assessed, n=5 (for F) and n=8 (for G), *= p<0.05, **= p<0.01, repeated measures 1-way ANOVA (for F), or Friedman test (for G). **(H-I)** BMDM from non-Tg or Tg mice were MI or challenged with Spn at MOI of 10 in the presence (+) or absence (-) of mT. 20 h post-challenge cells were assessed by nuclear morphology (H), n=3, *= p<0.05, **= p<0.01, 2-way ANOVA, or (I) lysates were probed for human (h) and murine (m) Mcl-1 expression by western blot and densitometry performed on three independent experiments. Data are represented as mean ±SEM. See also Figures E4-7.

Figure 4. Apoptosis-associated killing mediates bacterial clearance *in vivo.*

(A-E) CD68.hMcl-1 non-transgenic (non-Tg) or transgenic (Tg) mice were challenged with the designated dose of serotype 1 *Streptococcus pneumoniae* (Spn). 24 h after

instillation, bacterial colony forming units (CFU) in the lung homogenate (A) or blood (B), the number of polymorphonuclear leukocytes (PMN) (C), the number of alveolar macrophages (AM) (D), or the percentage of apoptotic AM (E) in the bronchoalveolar lavage (BAL) were measured, n=4-11 mice per group from three independent experiments, *= $p<0.05$ **= $p<0.01$, 2-way ANOVA. **(F-H)** Non-Tg and Tg mice were challenged with *H. influenzae* type b (Hib) at the designated dose. 24 h after instillation, CFU in the lung homogenate (F), PMN numbers in the BAL (G), and AM apoptosis (H), were measured, n=4-13 mice per group, from 2 independent experiments, ***= $p<0.001$, 2-way ANOVA. **(I-L)** Non-Tg or Tg mice were challenged with the designated dose of Spn intra-peritoneally. 24 h after challenge, the bacterial CFU in the peritoneal lavage (PL) (I), n=7, or blood (J), were determined, n=7, and PMN numbers (K), n=7 and levels of peritoneal macrophage (PM) apoptosis (L), n=9, in the PL were assessed by microscopy. In all experiments, *= $p<0.05$, **= $p<0.01$, 2-way ANOVA. See also Figure E8.

Figure 5: Apoptosis-associated killing can be reconstituted following challenge with *Streptococcus pneumoniae*

(A) Bone marrow-derived macrophages (BMDM) from CD68.hMcl-1 non-transgenic (non-Tg) or transgenic (Tg) mice were challenged with liposomes containing PBS (LIPO-PBS) or clodronate (LIPO-CLOD). At the designated time-point cells were fixed and analysed for nuclear fragmentation, n=3. **(B)** Wt or Tg BMDM were challenged with serotype 2 *S. pneumoniae* D39 (Spn) at a multiplicity of infection (MOI) of 10 in the presence of LIPO-PBS or LIPO-CLOD. 4 h post-challenge numbers of intracellular bacterial colony forming units (CFU) were assessed, n=3. **(C-D)** Non-Tg or Tg mice were infected with serotype 1 *S. pneumoniae* (Spn) in the presence of LIPO-PBS or LIPO-CLOD. Alveolar macrophage (AM) numbers in bronchoalveolar lavage (BAL) (C), and

AM apoptosis in BAL (D) were measured by microscopy 24 h post-challenge, both n=4, *= $p<0.05$, 2-way ANOVA. **(E-G)** Non-Tg and Tg mice were challenged with 10^5 serotype 1 Spn and liposome-encapsulated PBS (LIPO-PBS) or clodronate (LIPO-CLOD). 24 h after challenge, CFU in the lung (E) and blood (F), and total polymorphonuclear leukocyte (PMN) numbers in the BAL (G) were measured, n= 6-13 mice per group from 3 independent experiments. **(H-I)** Tg or non-Tg were instilled intranasally with 10^5 colony forming units of serotype 2 *S. pneumoniae* then immediately treated with ABT-263 or sabutoclax. 24 h after challenge, CFU in the lung (H) and blood (I) were measured (median + interquartile range), n= 8-10, *= $p<0.05$, unpaired Students t-test, or 2-way ANOVA, for analyses within or between groups respectively. See also Figure E9-10.

Figure 6*: Staphylococcus aureus* infection does not trigger apoptosis-associated killing.

(A) Bone marrow-derived macrophages (BMDM) from CD68.hMcl-1 non-transgenic (non-Tg) or transgenic (Tg) mice were challenged with *S. aureus* (Sa) at a multiplicity of infection (MOI) of 5. BMDM were lysed at varying time points during a 'pulse-chase' to allow detection of intracellular bacterial colony forming units (CFU), n=3. **(B)** BMDM apoptosis, in the same experiments, n=3. **(C)** BMDM were lysed for initial assessment of CFU or incubated in lysostaphin for 2 h, before CFU estimation to assess bacterial killing between the indicated time-points, n=3. **(D)** BMDM were challenged with Sa at MOI of 5 for varying time periods, extracellular bacteria killed and BMDM incubated with kanamycin and kanamycin resistant Sa, before extracellular bacteria were killed with lysostaphin and intracellular CFU measured at the designated time-points. The graph shows intracellular CFU, cultured in the presence of kanamycin to measure kanamycin resistant (recently ingested) bacteria over each time increment, n=3. **(E-G)**

Non-Tg or Tg mice were challenged with Sa at the designated dose. 24 h post-challenge, bacterial CFU in the lung homogenate (E), PMN numbers in the bronchoalveolar lavage (BAL) (F) and alveolar macrophage (AM) apoptosis (G), were measured, n=4-9 mice per group, from three independent experiments. See also Figure E11.